Generation of a $Na_V1.9$ overexpressing cell-line for screening compounds using automated patch-clamp

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Discovery from Charles River, Chesterford Research Park, Saffron Walden, UK

1 Introduction

Improving future pain management strategies to address the huge global health issue represented by a growing population of patients seeking medical care requires the development of better non-opioid analgesics.

$Na_V1.9$ is a voltage-gated sodium channel involved in pain signaling in the primary afferent sensory pathway in the peripheral nervous system. Genetic and pharmacological studies highlight the central role of $Na_V1.9$ in chronic and inflammatory pain states [1] highlighting it as a promising target for the discovery of much needed non-opioid analgesic pain therapeutics. High-throughput screening (HTS) assays are critical for identifying potent inhibitors of sodium channels and developing robust HTS assays enables the rapid identification of desirable selective inhibitors, accelerating the path towards potential novel pain medicines with fewer side effects.

Discovery efforts to screen against $Na_V1.9$ have been somewhat held back due to the unusually slow activation and inactivation kinetics of the channel, difficulties to establish robust overexpression in heterologous systems and the slightly more nuanced role of $Na_V1.9$ in pain signaling compared to other sodium channels.

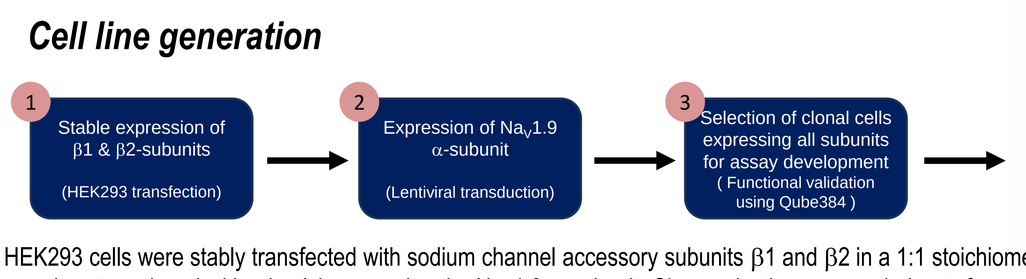
We discuss here the development of a HEK293 $Na_V1.9$ overexpressing cell line, its characterization and validation for implementation in a drug discovery screening cascade.

The data show sodium currents recorded from the recombinantly expressed ion channel have the expected biophysical properties and sensitivity to sodium channel blockers. Voltage-dependent inactivation of the currents enabled the measurement of state-dependent pharmacology that can be used to identify inhibitors that preferentially target the fast and

2 Methods

$Na_V1.9$ Qube assay development

Cell line generation



HEK293 cells were stably transfected with sodium channel accessory subunits β1 and β2 in a 1:1 stoichiometry. A stable pool was then transduced with a lentivirus carrying the $Na_V1.9$ α subunit. Clone selection was carried out after confirming stable transgene integration by RT-PCR and antibody staining.

Cells were cultured in complete growth media (DMEM with GlutaMax + 10% HI-FBS + 1x MEM NEAA) with selection for constitutive expression of $Na_V1.9$ (200 µg/mL Puromycin) and beta subunits (250 mg/mL G418).

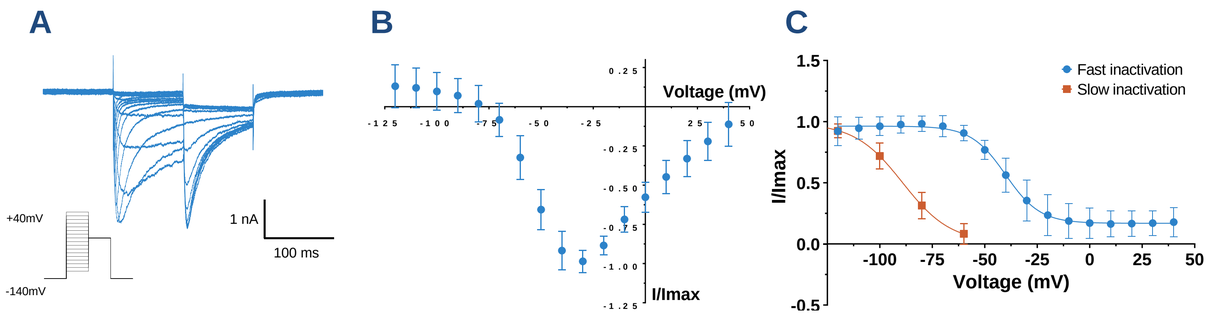
Electrophysiology was carried out on the Sophion Qube 384 well automated patch-clamp platform recording from 384 wells simultaneously. Assay performance was monitored across 10-plate stacker runs.

Intracellular solutions contained (in mM): CsF (120), CsCl (20),NaCl (10), HEPES (10), EGTA (10); pH 7.2 (CsOH); 310 mOsM and extracellular solutions contained NaCl (145), KCl (4), $MgCl_2$ (1), HEPES (10), $CaCl_2$ (3), glucose (10); pH 7.4 (NaOH); 320 mOsm. All experiments used multi-hole QChips. When used, Pluronic acid F-127 was applied at 0.01 % v/v.

Voltage protocols for measuring currents from the resting state used a depolarising test-step to -30 mV from a holding potential of -140 mV. Slow inactivated currents were evoked by a test-step to -30 mV from a -90 mV holding potential. 50% inactivation was induced after a 600 s recording period.

3 $Na_V1.9$ Cell-Line Characterisation and Validation

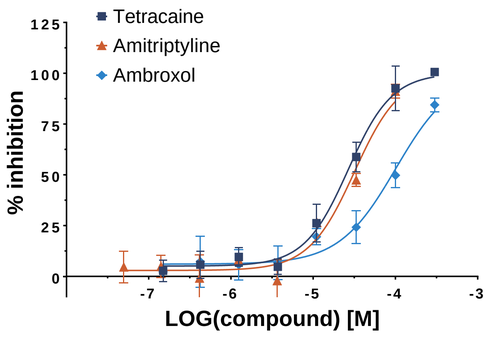
Biophysical properties



Currents recorded show characteristic $Na_V1.9$ biophysical properties

A. Representative current traces in response to the voltage-protocol shown on the bottom left. **B**. Current-voltage relationship showing average normalised peak current plotted against test potential. Peak of activation was found to be -30 mV. **C**. Voltage-dependence of the fast- and slowly-inactivated sodium current ($V_{half\text{-}fast}$ = -40.1 mV , $V_{half\text{-}slow}$ = -89.1 mV). The peak and voltage-dependence of activation and inactivation was in line with literature values[2].

Pharmacology

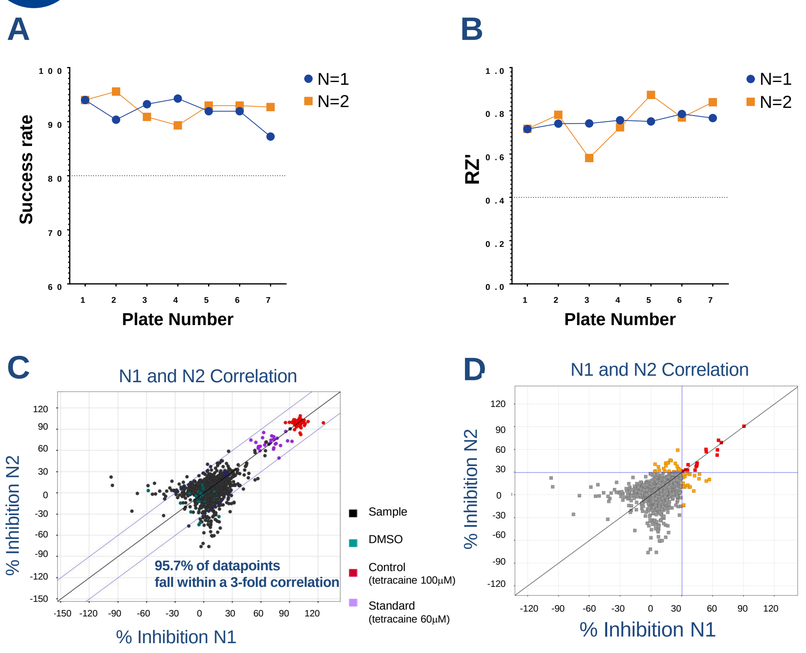


Compound	IC_{50} Resting state [µM]
Tetracaine hydrochloride	28.5
Amitriptyline hydrochloride	31.8
DSP-2230	17.2
Ambroxol	100.8
GDC-0276	22.7
Raxatrigine hydrochloride	35.0
Bupivacaine hydrochloride	112
Suzetrigine	>100
Lamotrigine	>100
TTX	> 1

Currents recorded show the expected $Na_V1.9$ pharmacological profile

A. Concentration-dependent responses to tetracaine, amitriptyline and ambroxol**. B**. Resting-state pharmacology was generated for a panel of known sodium channel blockers. Where available, IC_{50} values largely agree with reports in the literature[2]. Currents recorded were TTX-resistant up to µM concentrations providing further validation of the $Na_V1.9$ current.

4 Screening at scale for hit finding

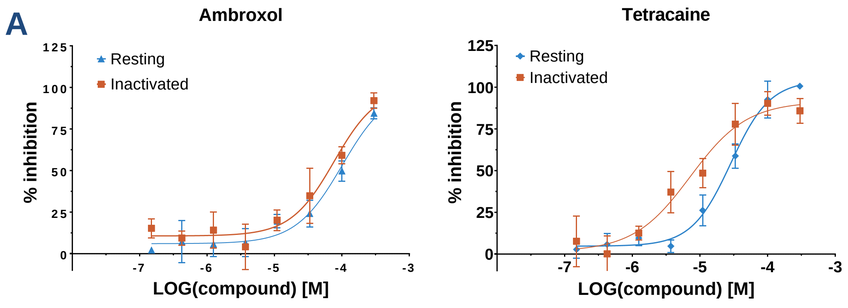


A pilot study shows a robust assay, which is scalable for HTS

7 plates (approximately 6,000 compounds) were tested as replicates at a single test concentration (10 µM).

A. Success rates across 7 plates were maintained above an assigned threshold (> 80%). Success rate was determined based on automatic filters (current expression >500 pA, membrane resistance >20 MΩ and capacitance >50 pF) and manual QC for wells that showed an effect. **B.** RZ' across 7 plates was maintained above a threshold of 0.4 **C.** Correlation plot of N1 and N2 show that 95.7% of data were within 3-fold. Controls are consistent across plates. **D.** Correlation plot of N1 and N2 show. Graph shows an arbitrary threshold of 30%, based on mean+3SD, is shown as lines in blue. Confirmed hits are shown in red, (~ 1% hit rate). Orange values show hits that were >30% in one replicate only.

5 State-dependent pharmacology



B

Compound	IC_{50} Resting state [µM]	IC_{50} Inactivated state [µM]
Tetracaine hydrochloride	28.5	7.2
Amitriptyline hydrochloride	31.8	15.24
DSP-2230	17.23	25.7
Ambroxol	100.8	76.48
GDC-0276	22.7	13.44
Raxatrigine hydrochloride	34.95	25.48
Bupivacaine hydrochloride	112	144
Suzetirgine	>100	>100
Lamotrigine	>100	>100

Secondary assay to determine state-dependent block

A voltage-protocol was developed to test separately the effect of compounds against the slowly inactivated state. Due to the very long recovery time (10 min) required for slowly inactivated $Na_V1.9$ channels, we could not develop a protocol to test resting- and slow-inactivation using one voltage-protocol. Compounds can be tested by applying two voltage-protocols in separate assays.

A. Concentration-dependent response to tetracaine and ambroxol. Tetracaine shows state-dependent block preferentially against the slowly-inactivated channel, while ambroxol shows similar potency against the resting- and slowly-inactivated state. **B.** IC_{50} values for resting and inactivated states are shown for a panel of known Na_V blockers.

Fast inactivated sodium currents could also be measured by using a brief depolarising pre-pulse calculated to inactivate exactly 50% of the current using the Qube's adaptive voltage control.

6 Conclusion

CRL has successfully generated a cell-line that expresses the human $Na_V1.9$ voltage-gated sodium channel

- Current recordings show characteristic $Na_V1.9$ biophysical properties including the slow activation, inactivation and insensitivity to TTX that gives rise to a persistent sodium current in dorsal root ganglion neurones
- The pharmacological profile of the currents are consistent across a panel of literature inhibitors tested

The cell line has been used to develop a primary functional assay suitable for HTS to identify $Na_V1.9$ inhibitors

- Pilot data show that the assay is robust and scalable and can identify potential novel hit matter from library size screening collections

State-dependent pharmacology can be measured in a secondary screening assay

- This can be used to show preferential binding against the deeply inactivated state as shown here by tetracaine

7 References

1. Brackx, W., Collaço, R. C., Theys, M., Vander Cruyssen, J., & Bosmans, F. (2023). Understanding the physiological role of $Na_V1.9$: Challenges and opportunities for pain modulation. *Pharmacology & Therapeutics*, *245*, 108416

2. Lin, Z., Santos, S., Padilla, K., Printzenhoff, D., & Castle, N. A. (2016). Biophysical and Pharmacological Characterization of $Na_V1.9$ Voltage Dependent Sodium Channels Stably Expressed in HEK-293 Cells. *PloS one*, *11*(8), e0161450

Acknowledgements

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For more information or to request a consultation with a specialist, please scan the QR code or visit www2.criver.com/ionchannel